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A novel electrochemical sensing strategy for rapid and ultrasensitive detection of *Salmonella* by rolling circle amplification and DNA–AuNPs probe

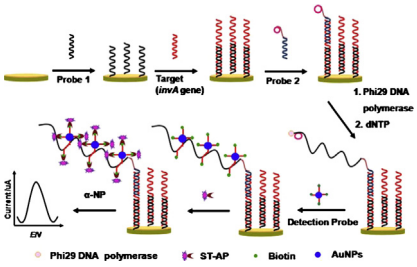
Dan Zhu [a,1], Yurong Yan [a,1], Pinhua Lei [a], Bo Shen [a], Wei Cheng [a,c], Huangxian Ju [a,b], Shijia Ding [a,*]

[a] Key Laboratory of Clinical Laboratory Diagnostics (Ministry of Education), College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, PR China
[b] State Key Laboratory of Analytical Chemistry for Life Science, Department of Chemistry, Nanjing University, Nanjing 210093, PR China
[c] The Center for Clinical Molecular Medical detection, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, PR China

HIGHLIGHTS

- This paper presented a novel sensing strategy for the rapid and ultrasensitive detection for *Salmonella*.
- Combination of rolling circle amplification and DNA–AuNPs probe is the first time for *Salmonella* electrochemical detection.
- The method displayed excellent sensitivity and specificity for detection of *Salmonella*.
- The fabricated biosensor was successfully applied to detect *Salmonella* in milk samples.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel electrochemical sensing strategy was developed for ultrasensitive and rapid detection of *Salmonella* by combining the rolling circle amplification with DNA–AuNPs probe. The target DNA could be specifically captured by probe 1 on the sensing interface. Then the circularization mixture was added to form a typical sandwich structure. In the presence of dNTPs and phi29 DNA polymerase, the RCA was initiated to produce micrometer-long single-strand DNA. Finally, the detection probe (DNA–AuNPs) could recognize RCA product to produce enzymatic electrochemical signal. Under optimal conditions, the calibration curve of synthetic target DNA had good linearity from 10 aM to 10 pM with a detection limit of 6.76 aM (S/N = 3). The developed method had been successfully applied to detect *Salmonella* as low as 6 CFU mL^{-1} in real milk sample. This proposed strategy showed great potential for clinical diagnosis, food safety and environmental monitoring.

1. Introduction

Salmonella,the gram-negative enteric bacilli, has been one of the most common bacteria responsible for foodborne diseases worldwide [1,2]. *Salmonella* is transmitted to humans mainly

* Corresponding author. Tel.: +86 23 68485688; fax: +86 23 68485786.
E-mail addresses: dingshijia@163.com, dingshijia@cqmu.edu.cn (S. Ding).
[1] These authors contributed equally to this work.

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Table 1
Sequences of the used oligonucleotides (in 5′–3′ direction).

Oligonucleotide	Sequence (5′–3′)
Forward primer	GCATCCGCATCAATAATACCG
Reverse primer	TTCTCTGGATGGTATGCCC
Probe 1	SH-$(CH_2)_6$-TTTTTTTTTAATACCGGCCTTCAAATCGGCATC
Probe 2	AATACTCATCTGTTTACCGGGCATAAAAAAAAAACACAGCTGAGGATAGGACAT
Target	ATGCCCGGTAAACAGATGAGTATTGATGCCGATTTGAAGGCCGGTATT
Non-complementary	AGCGCAGCTGCGCAATAGAATTGAAGAGGATTATGATGGCTACGTGAA
Circle template	P-CTCAGCTGTGTAACAACATGAAGATTGTAGGTCAGAACTCACCTGTTAGAAACTGTGAAGATCGCTTATTATGTCCTATC
Detection probe	SH-$(CH_2)_6$-TTTTTTTCAGAACTCACCTGTTAGTTTTTT-biotin

through the animal reservoir or contaminations of the food process line [3]. It is estimated that *Salmonella* causes 325,000 hospitalizations and 5000 deaths around 76 million cases of foodborne diseases yearly, according to World Health Organization (WHO) reports [4]. Therefore, it is extremely crucial to develop a method for the sensitive and rapid detection of *Salmonella*.

Several techniques have been used for the detection of *Salmonella*, such as conventional culture methods, enzyme-linked immunosorbant assay (ELISA) [5] and polymerase chain reaction (PCR) [6]. Conventional culture methods are reliable, but time-consuming (4–7 days) and labor-intensive [7,8]. The main disadvantages of ELISA are complicated wash procedure, labor-intensive analytical strategies and insufficient sensitivity [9]. PCR assays which employ visual scoring of the amplification products by ethidiumbromide-stained agarose gels are rapid, simple and more sensitive than ELSIA, but not enough to detect low concentrations of bacteria ($<10^5$ CFU mL^{-1}) [10]. In order to improve the detection of *Salmonella*, new biosensor-based methods have been developed as potential alternatives to break the bottlenecks of the conventional methods, because they have the distinct advantages of easy to use, rapid response, low cost and inexpensive instrumentation [11–13].

For the past few years, a variety of amplification strategies have been applied to improve the sensitivity of biosensors. Among the reported amplification strategies, rolling circle amplification (RCA) is one of the most popular amplification strategies. RCA, a simple isothermal DNA amplification process, can be used to generate a long ssDNA molecule with repetitive sequence units that are complementary to the circular DNA template, meaning that thousands of detection sites are generated from each template [14,15]. RCA possesses the advantages of simple and rapid steps, high potential and no requirement for special laboratory conditions, which made it a powerful tool over other amplification technologies [16–18]. So far, RCA has been widely used as an important technique for ultrasensitive DNA, RNA, and protein detection in diagnostic genomics and proteomics [19–22]. Meanwhile, gold nanoparticles (AuNPs) have also been widely used to further enhance sensitivity of sensors [23–25]. They are well-known as large specific surface area, unique physical–chemical properties, favorable biocompatibility and good conductivity [26–28].

Herein, to explore a rapid and ultrasensitive detection method of *Salmonella*, a novel strategy based on rolling circle amplification and gold nanoparticles was established. The *invA* is a highly conserved gene located on *Salmonella* pathogenicity island 1 (SPI-1) [29], which makes it be a potential target for *Salmonella* detection [10,30]. Combining the dual amplification strategies of RCA and DNA–AuNPs probe, this strategy could tremendously improve the sensitivity of *Salmonella* detection, which is conductive to the prevention and early diagnosis of foodborne disease.

2. Materials and methods

2.1. Reagents

DNA oligonucleotides were synthesized and purified by Sangon Inc., (Shanghai, China). Their sequences are listed in Table 1. 6-Mercapto-1-hexanol (MCH), streptavidin–alkaline phosphatase (ST-AP), α-naphthyl phosphate (α-NP), bovine serum albumin (BSA) and salmon sperm DNA were purchased from Sigma–Aldrich (USA). $HAuCl_4$ was purchased from Sinopharm Chem Ltd. (Shanghai, China). Phi29 DNA polymerase, T4 DNA ligase, and dNTP were purchased from Thermo (Waltham Mass, USA). Premix Taq Version 2.0, DL500 DNA Marker and agarose were purchased from Takara (Dalian, China). All other reagents were of analytical grade, and Millipore-Q water ($\geq$18 MΩ) was used in all experiments.

2.2. Apparatus

All electrochemical measurements were performed on a CHI 660D electrochemical workstation (Shanghai Chenhua Instruments Co., Ltd., China) with a conventional three electrode system composed of platinum wire as auxiliary, Ag/AgCl electrode as reference, and a 3 mm-diameter gold electrode as working electrode. UV–vis spectra were carried out on a UV 2550–vis spectrophotometer (Shimadzu, Japan). Transmission electron microscopic (TEM) image was carried out using an H-7500 transmission electron microscope (Hitachi, Japan). The PCR was carried out using a My Cycler thermal cycler (Bio-Rad Laboratories, USA). Gel images were recorded on an imaging system (Bio-Rad Laboratories, USA).

2.3. Preparation of AuNPs and DNA-functionalized AuNPs

AuNPs were prepared according to the literatures [31]. Briefly, 4 mL of 1% trisodium citrate was added to 100 mL of boiling 0.01% $HAuCl_4$ solution, stirring rapidly and boiling for 15 min. The solution turned deep red, indicating the formation of AuNPs. Then the resulting Au colloidal solution was cooled to room temperature with continued stirring and stored at 4 °C until use.

The DNA-functionalized AuNPs were achieved by adding 9 μL of 100 μM DNA probes into 300 μL of AuNPs solution [32]. After incubating for 12 h at 4 °C with slight stirring, the DNA–AuNPs conjugates were "aged" in 0.5 M NaCl for another 12 h. Finally, the solution was centrifuged at 12,000 rpm for 30 min to remove the excess reagents. The red precipitate was washed, centrifuged and dispersed in hybridization buffer for future use.

2.4. Preparation of DNA samples and PCR amplification

Salmonella typhimurium strains were obtained from Chongqing Municipal Center for Disease Control and Prevention. These strains were grown at 37 °C for 16 h in sterile liquid Luria–Bertani medium with shaking. The culture was ultracentrifuged at 12,000 rpm for

10 min to be further purified and resuspended in sterile ultrapure water. The enumeration of viable *Salmonella* was carried out by plating 100 μL of appropriate 10-fold dilutions in sterile ultrapure water onto plate count agar. After incubating the plates at 37 °C for 24 h, the culture colonies on the plates were counted to estimate the number of viable cells in CFU mL^{-1}.

For the detection of real samples, skimmed milk purchased in local commerce area was spiked with *Salmonella* at the different concentrations which were boiled for 15 min at 100 °C in a water bath and immediately chilled on ice [33]. After centrifugation at 10,000 rpm for 5 min at 4 °C to eliminate lipids and proteins, the supernatant containing genome DNA was transferred to a new tube, used as PCR template. All DNA preparations were stored at −20 °C prior to use.

The PCR reactions were performed in 50 μL volumes consisting of: 5.0 μL genomic DNA, 1.0 μL of 20 μM forward and reverse primers, 25 μL of Premix Taq (1.25 U of DNA polymerase, 2× Taq buffer, 0.4 mM of dNTPs) and 18 μL of water. The cycling parameters consisted of 35 cycles of denaturation at 95 °C (30 s), annealing at 51 °C (30 s) and extension at 72 °C (30 s) followed by a final extension at 72 °C for 4 min. PCR products were determined by running 10 μL of PCR mixtures in 3–4% agarose gel for 20 min and observed under ultraviolet light.

2.5. Circularization of DNA template

Hundreds nanomoles of circular template oligonucleotide and 100 nanomoles of biotinylated primer oligonucleotide were mixed in 100 μL of ligation buffer (50 mM, pH 7.5 Tris–HCl buffer, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM ATP). Then, 1 unit of T4 DNA ligase was added and incubated at 37 °C for 1 h. After ligation, T4 DNA ligase was inactivated by heating the reaction mixture at 65 °C for 10 min. The resulting mixture could be used directly or stored at −20 °C.

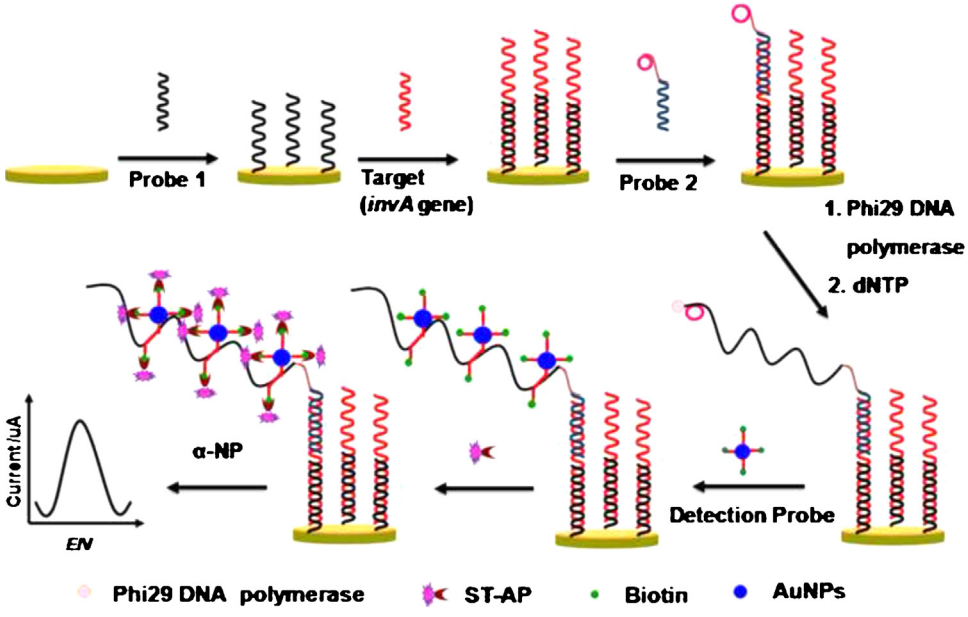
2.6. Preparation of electrochemical biosensor

The bare gold electrode was polished with 0.05 μm alumina slurries and ultrasonically treated in ultrapure water for a few minutes, followed by soaking in piranha solution (H_2SO_4:H_2O_2 = 3:1) for 10 min to eliminate other substances. Then, the pretreated electrode was rinsed with ultrapure water and allowed to dry at room temperature. 10 μL of 100 nM thiolated capture probe was dropped onto the pretreated gold electrode surface and incubated overnight at 4 °C. After washing with washing buffer, the electrode was treated with 1 mM MCH for 1 h to obtain well-aligned DNA monolayer and occupy the left bare sites [24,33], and further immersed in salmon sperm DNA and 1% BSA for 30 min respectively to avoid nonspecific adsorption of DNA and enzyme on the electrode surface. The PCR product was denatured by heating at 100 °C for 5 min in a water bath, and immediately chilled in ice for 5 min to obtain denatured ssDNA before the detection. The synthetic target DNAs were diluted to the desired concentration. The fabricated biosensor firstly hybridized specifically with target DNA for 1 h at 37 °C. After the biosensor was thoroughly washed with washing buffer, 10 μL of circularization mixture containing 20 nM circular template DNA and 20 nM biotinylated primer DNA was dropped onto biosensor surface and incubated for 1 h at 37 °C. Following rinsed thoroughly with washing buffer, RCA reaction was initiated by addition of 0.5 units of phi29 DNA polymerase in 10 μL of reaction buffer (50 mM, pH 7.5 Tris–HCl buffer, 10 mM magnesiumacetate, 33 mM potassium acetate, 1 mM dithiothreitol, 10 mM dNTP, and 0.1% Tween 20) and continued for 1 h at 37 °C. Then the biosensor was carefully washed with washing buffer, 10 μL of DNA–AuNPs detection probe was dropped onto biosensor and hybridized at 37 °C for 1 h. After the biosensor was rinsed with DEA buffer, 10 μL of 0.5 μg mL^{-1} ST-AP was dropped onto its surface and incubated at 37 °C for 30 min. Finally the biosensor was washed with diethanolamine buffer containing 0.05% Tween-20 thoroughly to perform differential pulse voltammetry (DPV) detection in diethanolamine buffer containing 0.75 mg mL^{-1} of α-NP.

3. Results and discussion

3.1. Design of electrochemical biosensor

Scheme 1 depicts the biosensing process of *Salmonella* detection. The target DNA firstly hybridized with specifically designed capture DNA. Then the circularization mixture was added



Scheme 1. Schematic representation of the designed strategy for *invA* detection of *Salmonella*.

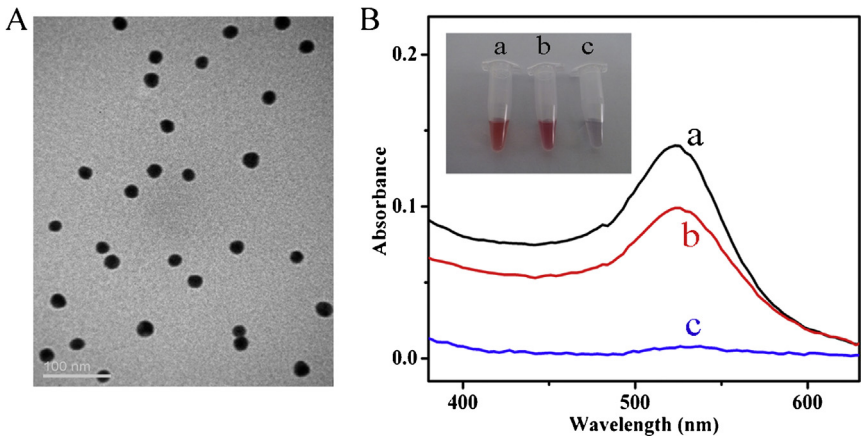


Fig. 1. (A) TEM image of AuNPs. (B) UV–vis absorption spectra of (a) AuNPs, (b) AuNPs with SH-DNA after addition of 0.5 M NaCl, (c) AuNPs without SH-DNA after addition of 0.5 M NaCl.

to form a typical sandwich structure. In the presence of dNTPs and phi29 DNA polymerase, the RCA was initiated to produce micrometer-long single-strand DNA, which contained hundreds of tandem-repeat sequences for the attachment of a large number of DNA–AuNPs probes for enhancement of recognition event (Table 1). Finally, the resulting AuNPs-tagged RCA product was linked to ST-AP to produce enzymatic electrochemical signal readout for quantitative detection of *Salmonella*. The dual signal amplification strategies provided a novel sensing platform for ultrasensitive detection of *Salmonella*.

The TEM image of the synthesized AuNPs is shown in Fig. 1A. It displayed spherical and homogeneous dispersion with an average diameter of 18 nm. The DNA–AuNPs conjugates were confirmed by UV–vis absorption spectra (Fig. 1B), which demonstrated that the successful labeling of thiol-modified oligonucleotides was achieved.

3.2. Characterization of biosensor fabrication

Electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV) measurement were used to characterize the electrochemical DNA biosensor (Fig. 2A). In the terms of EIS, $[Fe(CN)_6]^{3-/4-}$ was utilized as the redox probe and the semicircle diameter was equal to electron-transfer resistance, Ret. The bare electrode exhibited an almost straight line (curve a), which was characteristic of a mass diffusion limiting step of the electron-transfer process. When the capture DNA was self-assembled onto the bare electrode, the Ret increased (curve b). This was because that the negatively charged phosphate backbone of the oligonucleotides produced an electrostatic repulsion force to $[Fe(CN)_6]^{3-/4-}$. The Ret further increased (curve c), after the biosensor was hybridized with target DNA. When RCA reaction was finished, the Ret increased significantly (curve d), which proved the successful implement of RCA reaction. Afterwards, upon the hybridization of the DNA–AuNPs probe with the RCA products, the Ret decreased significantly (curve e), which was attributed to the fact that nanomaterials had large specific surface area and could accelerate the electron transfer. These results were in a good agreement with those obtained from SWV measurements (Fig. 2B), in which the peak currents varied upon the assembly and binding processes. Both results of EIS and SWV proved that the biosensor worked indeed as described in the principle scheme.

3.3. Signal amplification performance of designed biosensor

In this strategy, AuNPs had a very important role in signal amplification. The DPV response of 100 fM target in the presence of AuNPs was much larger than that in the absence of AuNPs due to the AuNPs – assisted electron transfer (Fig. 3). Hence, the biosensing platform was established for the specific and ultrasensitive determination of *Salmonella*.

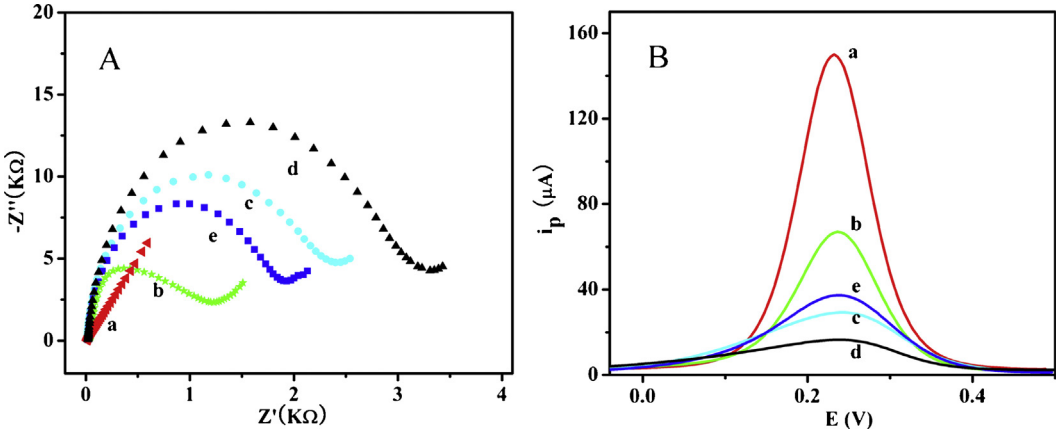


Fig. 2. EIS (A) and SWVs (B) in 0.4 M KCl containing 0.5 mM $Fe(CN)_6^{3-/4-}$ at bare electrode (a), capture DNA modified electrode (b), capture DNA modified electrode after hybridized with target DNA (c), after RCA (d) and further reaction with DNA–AuNPs (e).

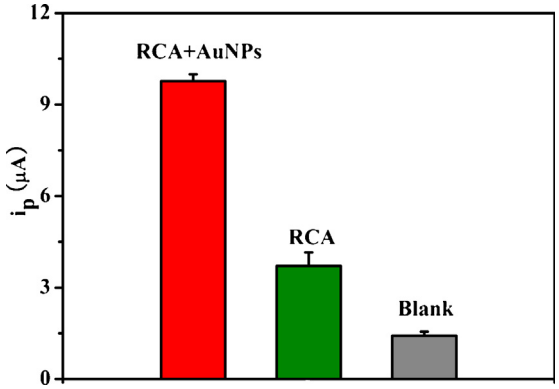


Fig. 3. Comparison of DPV peak currents in the absence (blank) and the presence of 100 fM target with RCA (without AuNPs) and RCA–AuNPs.

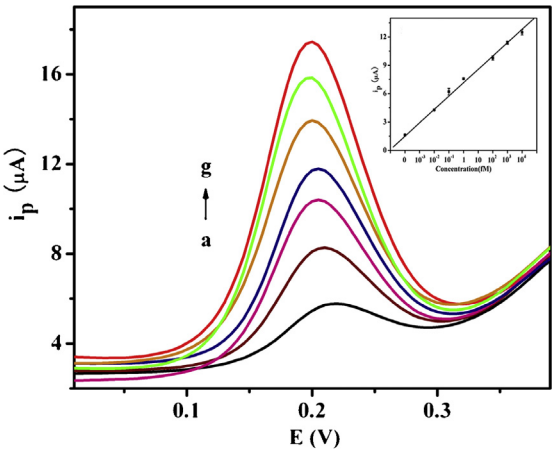


Fig. 5. DPV response to 0, 0.01, 0.1, 1, 100, 1000, 10,000 fM target DNA (from a to g). Inset: Calibration plot of DPV peak current vs logarithm of target DNA concentration. The error bars represent the standard deviations in three different measurements for each concentration.

3.4. Optimization of experimental conditions

For the sake of achieving the excellent assay performance, the different experimental conditions were optimized. The amounts of circularization mixture acting as a primer to initiate a RCA reaction greatly affected the biosensor performance. Therefore, the concentration of circularization mixture was firstly optimized. With the increasing concentration of circularization mixture, the DPV response rose gradually and then tended to a constant value at 20 nM (Fig. 4A). So 20 nM circularization mixture was used in all subsequent experiments.

The time of RCA process also played a very important role in this experiment. At the circularization mixture concentration of 20 nM, the signal increased gradually with the time of RCA reaction and reached stable value at 60 min (Fig. 4B). Therefore, 60 min was adopted as the optimal RCA reaction time. As shown in (Fig. 4C), the DPV peak current increased sharply and the signal exhibited no further remarkable variation after more than 0.75 mg mL^{-1} α-NP. Hence, the optimized concentration of α-NP was determined to be 0.75 mg mL^{-1} for the following work.

3.5. Analytical performance of designed biosensor

3.5.1. Sensitivity of designed biosensor

Under the optimal experimental conditions, the DPV responses for synthetic target oligonucleotides at different concentrations were shown in Fig. 5. It was found that the DPV peak current increased with the increasing concentration of target oligonucleotides. In order to investigate the analysis capability of this designed method, the dynamic range for detection of target DNA concentration was examined. The inset showed the good linear relationship between the DPV responses and the logarithm of target DNA concentration in the range of 10 aM to 10 pM. The resulting linear equation was I (μA) = 0.14 × lg c + 0.72 (c is the concentration of target DNA (fM)) with a correlation coefficient of 0.9972. Additionally, the limit of detection (LOD) was 6.76 aM at a signal-to-noise ratio of 3, which was much lower than previous reported methods (Table 2) [2,10]. The achieved ultrasensitivity could be attributed to dual signal amplifications of RCA and AuNPs.

3.5.2. Specificity and reproducibility of the assay

To investigate the specificity of the biosensor to different oligonucleotides, full-complementary oligonucleotides and non-complementary oligonucleotides were analyzed. Fig. 6 displayed the changes of DPV after hybridization with 100 fM and 10 pM of the two different oligonucleotides and the background. The DPV responses of full-complementary oligonucleotides were much larger than those of non-complementary oligonucleotides and blank, which demonstrated that the designed biosensor could effectively discriminate different DNA sequences and displayed excellent selectivity. To evaluate the repeatability of the developed biosensor, the synthetic target DNA at 1 pM was examined 5 times. The relative standard deviation was less than 5%, which manifested that this method had an acceptable reproducibility.

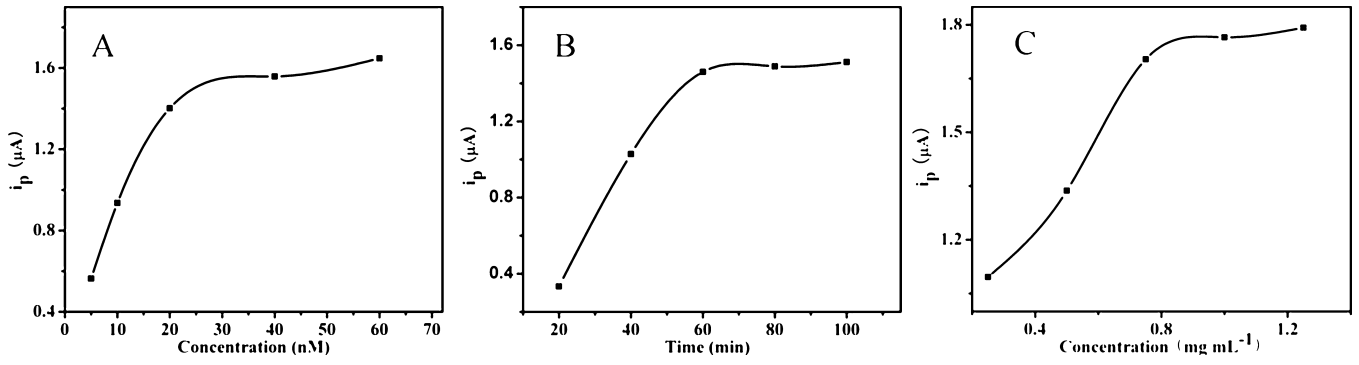


Fig. 4. Dependences of DPV peak currents on circularization mixture concentration (A), RCA reaction time (B), α-NP concentration (C), when one parameter changes while the others are under their optimal conditions.

Table 2
Comparison between the proposed method and other reported biosensors for detection of Salmonella.

Biosensor platform	Bio-receptor of immobilization	LOD (L^{-1})	LOD (CFU mL^{-1})	Ref.
Fluorescence	Oligonucleotide	3 fmol	30	[2]
SPR	Oligonucleotide	0.5 mol	10^2	[10]
Magnetoelastic	E2 phage	–	5×10^2	[34]
Fiber-optic	Antibody	–	10^3	[35]
Electrochemical (chronoamperometry)	Antibody	–	5×10^3	[12]
Electrical impedance	Antibody	–	10^3	[36]
Screen-printed carbon electrode	Antibody	–	143	[13]
Electrochemical (DPV)	Oligonucleotide	6.76 amol	6	This study

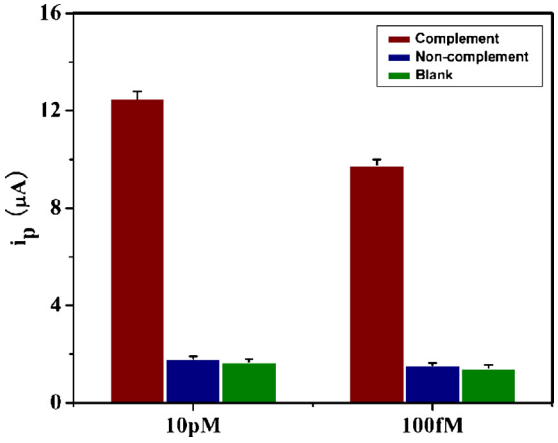


Fig. 6. Comparison of DPV peak currents after hybridization with 10 pM and 100 fM of target oligonucleotides, non-complementary oligonucleotides and blank.

3.6. Detection of Salmonella in real samples

To evaluate the feasibility of the fabricated biosensor for the detection of actual samples, skimmed milk purchased in local commerce area was spiked with Salmonella at different concentrations. PCR was then carried out using the genomic DNA extracted from each concentration of *Salmonella* in skimmed milk. The agarose gel electrophoresis detection of PCR products with the right size (75 bp) was shown in Fig. 7A. The gel electrophoresis could not effectively identify the PCR products for *Salmonella* less than 10^5 CFU mL^{-1} due to the low EtBr straining efficiency for ssDNA. Meanwhile, the established electrochemical DNA biosensor was also applied to analyze the denatured PCR products for *Salmonella* (0 to 6×10^8 CFU mL^{-1}). The responses of the biosensor to different PCR samples were shown in Fig. 7B. The inset of Fig. 7B showed the good linear relationship between the DPV responses and the logarithm of *Salmonella* concentration (6 to 6×10^5 CFU mL^{-1}), with a correlation coefficient of 0.9989. Furthermore, the fabricated DNA biosensor could detect *Salmonella* concentration as low as 6 CFU mL^{-1} in real milk samples, which was lower than other methods reported previously for the detection of *Salmonella* (Table 2) [13,34–38].

To investigate the specificity of the established electrochemical DNA sensor, five PCR products for different types of bacteria were assayed (Fig. 7C). The PCR products were 6×10^8 CFU mL^{-1} of *Salmonella* (a), Escherichia *coli* (b), *staphylococcus aureus* (c), *Streptococcus pneumoniae* (d) and blank (e) respectively. The DPV response of (a) was much larger than those of (b)–(e), while the signals of (b)–(d) were close to that of (e), these findings demonstrated that the designed biosensor displayed excellent specificity for the detection of *Salmonella*. The high specificity and sensitivity could further ensure the practicality of the developed biosensor.

4. Conclusions

In summary, an ultrasensitive and rapid biosensor was developed for detection of *Salmonella* by combining the rolling circle amplification technique with DNA–AuNPs probe. The amplification strategy greatly improved the sensitivity for detection of a complementary target DNA down to 6.76 aM. Also, the designed assay had been successfully applied to detect PCR amplified products from *Salmonella* and could detect *Salmonella* as low as 6 CFU mL^{-1} in real milk samples. This biosensing strategy could provide a simple, versatile, low cost and powerful platform for *Salmonella* screening in clinical diagnosis, food safety, biothreat detection and environmental monitoring.

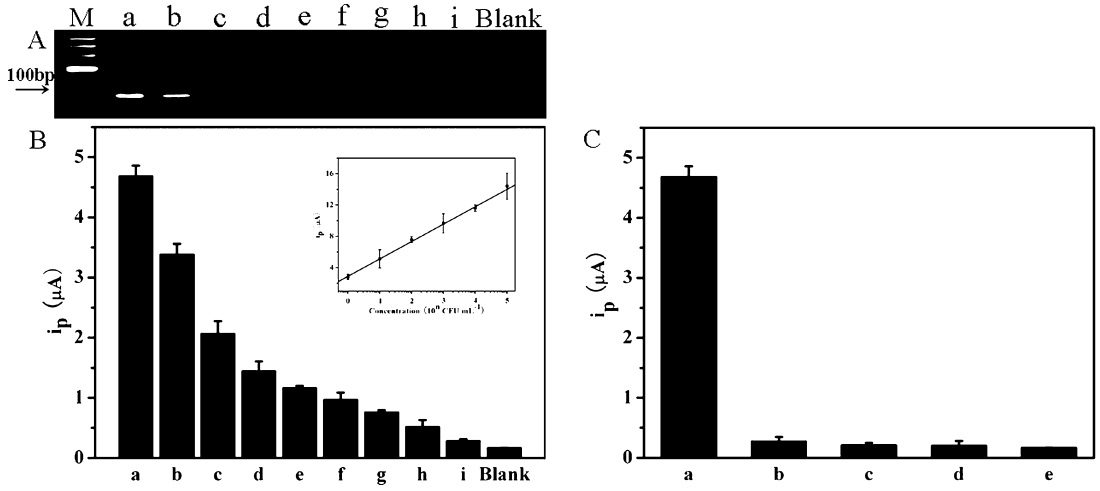


Fig. 7. (A) Gel electrophoresis photos of 500 bp size maker (M), and PCR products of 6×10^8 (a), 6×10^7 (b), 6×10^6 (c), 6×10^5 (d), 6×10^4 (e), 6×10^3 (f), 6×10^2 (g), 6×10^1 (h), 6 (i) CFU mL^{-1} of *Salmonella*. (B) DPV peak currents responding to PCR products obtained from serial dilutions of *Salmonella* in the range of 0 to 6×10^8 CFU mL^{-1}. Inset: Plot of DPV peak current vs logarithm of *Salmonella* concentration. (C) DPV peak currents responding to PCR products of 6×10^8 CFU mL^{-1} of *Salmonella* (a), *E. coli* (b), *staphylococcus aureus* (c), *Streptococcus pneumoniae* (d), and blank (e).

Acknowledgments

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